

Overexpression of P53 protein and local hGH, IGF-I, IGFBP-3, IGFBP-2 and PRL secretion by human breast cancer explants

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Abstract

BACKGROUND: Insulin-like growth factor-I (IGF-I) in concert with insulin-like binding protein 3 (IGFBP-3), insulin-like binding protein 2 (IGFBP-2), human growth hormone (GH) and P53 protein is involved in autocrine/paracrine growth signaling pathways as an adaptive response to environmental stimuli. **OBJECTIVE:** The study evaluated the local secretion of PRL, hGH, IGF-I, IGFBP-2 and IGFBP-3 by breast cancer tissue explants in relation to the overexpression of P53 protein in breast cancer tissue. **MATERIALS AND METHODS:** Breast cancer explants were obtained during radical mastectomies. The overexpression of P53 protein was assessed immunohistochemically using monoclonal antibody (DAKO, Anti-Human P53 protein, clone DO-7); the results of the reaction were stratified into 5 groups. The lack of P53 protein overexpression was defined as 0% of cells that overexpressed P53 protein. IGF-I, IGFBP-3, IGFBP-2, and hGH levels were measured with RIA kits, and prolactin was measured with the MEIA kit. **RESULTS:** The local secretion of hGH by tumour explants – presenting a positive immunohistochemical reaction (IHCR) to the product of P53 gene – was twice as high as those with no IHCR to product of P53 gene; the opposite was noted in the case of IGF-I, IGFBP-2 and IGFBP-3 secretion. In both cases, the level of hGH, IGF-I and IGFBP-3 secretion did not correlate with the ratio of cells overexpressing P53 protein. There was a significant decrease in local, basic IGFBP-2 secretion along with an increased ratio of cells with positive IHCR to product of P53 gene. Furthermore, local PRL secretion was not correlated with the ratio of cells overexpressing P53 protein in breast cancer tissue. Prolactin also exerts no influence on IGF-I secretion. **CONCLUSION:** Our results may suggest the presence of local hGH/IGF-I feedback in breast tissue as well as the possibility of P53/hGH/IGF-I/IGFBP-3 but not P53/PRL/IGF-I axis.

INTRODUCTION

Cellular survival and proliferation can be positively regulated through the action of cytokines. The signaling effects of P53 and cytokines on the cellular phenotype are counteracting (Fritsche *et al.* 1998). Tumour suppressor genes function as the specific inhibitors of tumour cell growth. Inhibition of tumour cell proliferation by P53 is exerted by means of activation of tumour cell differentiation (Li *et al.* 1998). Tumour suppressor P53 is a phosphoprotein barely detectable in the nucleus of normal cells and is also regarded as a nuclear transcription factor that inhibits cell cycle progression and induces apoptosis (Fritsche *et al.* 1998; Benchimol *et al.* 1982; Ryan & Vousden 1998; Werner *et al.* 1996). P53 gene is the single most important human tumour suppressor that is commonly affected by mutations in human malignancies including breast cancer (Werner *et al.* 1996; Grimberg 2000). Experimental data has shown that cells containing the wild-type P53 gene undergo death by apoptosis after anticancer treatment, whereas cells that lack wild-type P53 gene show resistance to drugs (Lowe *et al.* 1993). In times of cellular stress, wild-type P53 gene can arrest cell cycle progression, thereby allowing DNA to be repaired (Kastan *et al.* 1991). If the damage is beyond cell repair capability, the P53 protein diverts the fate of the cell into apoptosis (Ferreira *et al.* 1999). Wild type P53 tumour suppressor is a crucial mediator of apoptosis in response to cellular stress (Butt *et al.* 1999). According to Niezabitowski *et al.* p53 protein may be considered one of the independent prognostic parameters for disease free survival of patients with phyllodes tumour of the breast (Niezabitowski *et al.* 2001). The College of American Pathologists included the p53 gene and its protein product in the group of breast cancer prognostic factors, which should be investigated intensively (Fitzgibbons, *et al.* 2000). The mode of activation of many tumor suppressor genes involves transcriptional suppression of IGF-I receptor gene with subsequent reduction of cell surface receptor concentration and IGF-I action. A loss of function mutation of tumor suppressors can lead to aberrant regulation of IGF-I receptor gene expression (Werner & Maor 2006). p53 gene apoptotic action is exerted via manipulation of insulin-like growth factor (IGF) axis components (Grimberg 2000). Wild type P53 gene induction causes the down-regulation of membrane IGF-I receptor. The mutant form of P53 gene is important for up-regulation of the receptor (Girnita *et al.* 2000). IGF-I in concert with insulin-like binding protein-3 and P53 is involved in autocrine/paracrine growth signaling pathways as adaptive responses to environmental stimuli (Kari *et al.* 1999). Webster *et al.* and Ohlsson *et al.* found a positive correlation between expression of P53 and IGF-I receptor expression (Webster *et al.* 1996; Ohlsson *et al.* 1998). Transcriptional activation of IGF-I receptor gene requires an intact P53 signaling pathway (Glait *et al.* 2006). The addition of

IGF-I to *in vitro* mouse mammary gland whole organ culture – that was exposed to estrogen and progesterone – inhibited cell death and expression of P53 (Mathews & Schneider 2008).

Carcinogenesis involves an accumulation of specific molecular alterations occurring in specific proto-oncogenes or tumour suppressor genes. Rapid cellular turnover may increase the rate at which the alterations accumulate. The IGF axis is one of the determinants of cellular turnover rate (Baserga 1995). Appropriate levels of P53 activity are important to the regulation of mammary gland ductal morphogenesis in part through regulation of the IGF-I pathway (Gatza *et al.* 2008). The insulin-like growth factors and its binding proteins have been disturbed in breast cancer (Landberg *et al.* 2001). The mutant form of P53 gene loses its apoptotic activity and ability to activate transcription from the P53 protein responsive portion of Insulin-like growth factor binding protein-3 (IGFBP-3) gene promoters (Friedlander *et al.* 1996). IGFBP-3 inhibits cell growth and enhances apoptosis not only by limiting the availability of IGF-I to its receptors but also independently by its own receptors (Hollowood *et al.* 2000; Oh 1998). The presence of IGF/P53 axis may be suggested (Webster *et al.* 1996).

Our previous studies showed the local secretion of hGH, IGF-I and prolactin by human breast cancer explants. Moreover, the studies demonstrated that hGH stimulates local IGF-I secretion by human breast cancer explants expressing progesterone receptors. Progesterone induced the decrease of IGFBP-3 secretion in the same type of malignant explants. Therefore, it seemed interesting to evaluate the relationship between local PRL, hGH and IGF-I secretion from human breast cancer explants and the expression of the P53 protein mutant form.

MATERIALS AND METHODS

Tissues

Breast cancer explants were obtained during 48 radical mastectomies performed at the Cracow Branch of National Institute of Oncology Cracow, Poland. Explants of malignant tissue were prepared under close supervision of a pathologist, eliminating the hazard of interfering with further pathologic evaluation of a malignant lesion of the breast. Some of the specimens were fixed in 10% buffered formalin for 48 hours at room temperature, dehydrated and embedded in paraffin by a standard procedure. A section 5- μ m thick was mounted on slides and deparaffinated before processing for receptor determination by immunohistochemistry. The overexpression of P53 protein was evaluated by monoclonal antibodies from commercial kits manufactured by the DAKO Company (DAKO, Anti-Human p53 protein, clone DO-7). The results of the evaluation were stratified into 5 groups: lack of P53 overexpression (0% of cells immunohistochemically positive to P53),

weak positive overexpression (0–10% of cells immunohistochemically positive to P53), *moderate* overexpression (10–50% of cells immunohistochemically positive to P53), *strong* overexpression (50–80% of cells immunohistochemically positive to P53) and *very strong* overexpression (80–100% cells immunohistochemically positive to P53).

Mammary gland in vitro incubation

Primary tumour explants were prepared according to Gregoraszczuk. Briefly, remaining explants from the malignant tissue and surrounding non-malignant tissue were trimmed into 0.5 cm³ pieces, weighed and incubated in 2 ml of M199 medium containing 5% of calf serum. Afterward, results obtained by means of immunohistochemistry were matched to those from culture. Four explant pieces obtained from each malignant tissue of each individual patient were put into culture tubes and incubated for 48hrs in a medium. Experimental tubes with explants were shaken gently at 37°C. The media was continuously gassed with 5% CO₂/air during incubation. After 48 hours incubation, the collected media were frozen at –20°C for evaluation of hGH, PRL, IGF-I, IGFBP-3 and IGFBP-2 level in the media.

Hormone concentration evaluation

IGF-I level was measured, using Biosource Europe S.A. RIA kits sensitivity 0.25 ng/ml; the coefficients of variation within and between assays were 4.1% and 9.3% respectively; cross reaction, with insulin 0.001%, with hGH 0.01%, and with IGF-II 0.2%. Human growth hormone (hGH) level was estimated with OBRI POLATOM (Świerk, Poland), RIA kits: sensitivity 0.5 ng/ml, the coefficients of variation within, and between, assays were 2.1% and 2.7% respectively. For prolactin assessment an Abbot Lab MEIA (Microparticle Enzyme Immunoassay) kit was used: sensitivity 0.6 ng/ml, cross reaction with hGH 0.039%, and no cross reaction with FSH, LH, TSH, hCG, hPL.

IGFBP-3 level was measured by RIA kits manufactured by Diagnostic Systems Laboratories, Webster,

Texas, USA. The sensitivity was 0.5 ng/ml, whereas the specificity was 99%. IGFBP-2 level was evaluated also by RIA kits manufactured by Diagnostic Systems Laboratories, Webster, Texas, USA. The sensitivity was 0.5 ng/ml, whereas the specificity was 99%. No cross reactivity was found with hGH, IGF-I, IGF-II, IGFBP-3, IGFBP-4, IGFBP-5, IGFBP-6.

IGFBP-2 level was measured by RIA kits manufactured by Diagnostic Systems Laboratories, Webster, Texas, USA. The sensitivity was 0.5 ng/ml, whereas the specificity was 99%. No cross reactivity was found with hGH, IGF-I, IGF-II, IGFBP-3, IGFBP-4, IGFBP-5, IGFBP-6.

Statistical methods

All data points are expressed as means \pm SEM. A significant difference in hGH, prolactin, IGF-I, IGFBP-3 and IGFBP-2 levels in malignant tissue also showed diverse overexpression of P53; comparison was made by analysis of variance and Duncan's new multiple range test.

RESULTS

The lowest [$p < 0.01$] secretion of hGH was found in explants with a lack of cells showing positive immunohistochemical reaction (IHCR) to P53 protein [0.18 ± 0.04 ng/10mg tissue]. Explants with various rates of cells showing positive IHCR to P53 protein showed higher secretion of hGH but without significant differences between groups of P53 overexpression levels [0.54 ± 0.02 ng/10 mg tissue; 0.51 ± 0.1 ng/10 mg tissue; 0.54 ± 0.05 ng/10mg tissue; 0.61 ± 0.1 ng/10mg tissue respectively in 0–9.9%; 10–49.9%; 50–79.9%; 80–100% of cells with positive IHCR for P53 protein (Figure 1).

The highest local IGF-I secretion was observed in explants with a lack of immunohistochemical reaction (IHCR) to P53 protein. The secretion by explants mirrored in a way the hGH secretion pattern. The explants, which showed negative IHCR to P53 protein, secreted the highest amount of IGF-I [24.14 ± 0.24 ; 24.91 ± 0.19 ng/10 mg tissue]. The explants which showed positive IHCR to P53 protein secreted significantly ($p < 0.05$) lower amounts of IGF-I [14.21 ± 0.8 ng/10 mg tissue; 14.3 ± 2.4 ng/10 mg tissue; 14.0 ± 2.4 ng/10 mg tissue respectively in 0–10%; 10–50% and 50–80% cells with P53 protein IHCR positive explants vs. 24.1 ± 4.0 ng/10 mg tissue in explants with lack of cells with P53 protein IHC positive reaction. The lowest secretion of IGF-I [10.6 ± 1.05 ng/10mg of tissue] was observed in explants showing the highest rate (80–100%) of cells with positive IHCR to P53 protein (Figure 2).

The local secretion of IGFBP-3 was the lowest by explants with the highest rate of P53 protein IHC positive cells (80–100%) [8.8 ± 1.0 ng/10mg of tissue]. The secretion of IGFBP-3 was higher by explants with 50–79.9% of P53 protein IHC positive cells [11.1 ± 1.3 ng/10 mg of tissue vs. 8.8 ± 1.0 ng/10mg of tissue; $p < 0.05$]. The secretion of IGFBP-3 was the highest by

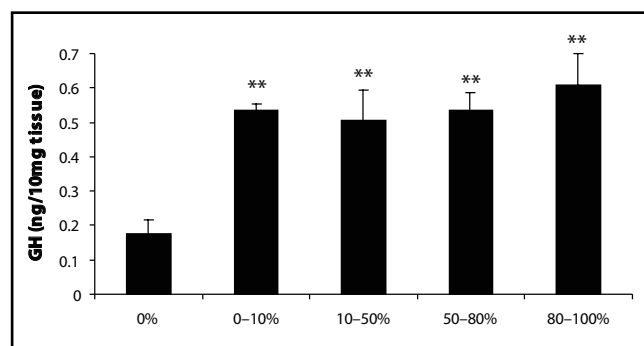


Fig. 1. Secretion of hGH by breast cancer explants in relation to ratio of cells with showing positive immunohistochemical reaction (IHCR) to P53 protein (** $p < 0.01$).

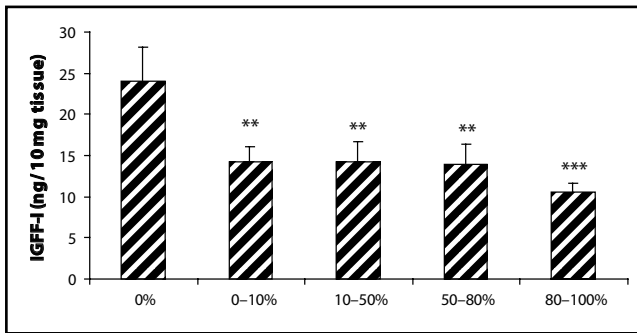


Fig. 2. Secretion of IGF-I by breast cancer explants in relation to ratio of cells with showing positive immunohistochemical reaction (IHCR) to P53 protein (** $p<0.05$).

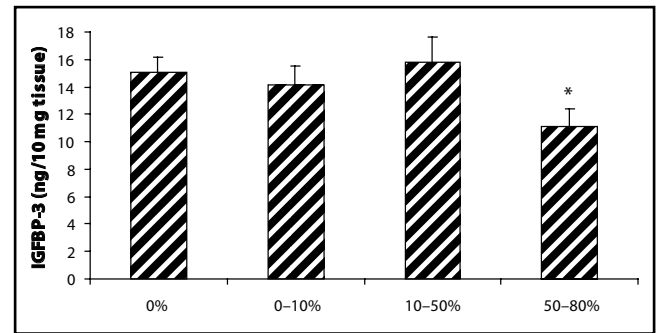


Fig. 3. Secretion of IGFBP-3 by breast cancer explants in relation to ratio of cells with showing positive immunohistochemical reaction (IHCR) to P53 protein (* $p<0.05$).

explants with ratio of P53 IHC positive cells lower than 50% [15.1 ± 1.1 ng/10mg of tissue; 14.1 ± 1.4 ng/10 mg of tissue; 15.8 ± 1.8 ng/10 mg of tissue respectively in 0%; 0–9.9%; 10–49.9% of cells with positive IHCR for P53 protein (Figure 3).

The pattern of local IGFBP-2 secretion by explants with various rates of P53 IHC positive cells similarly follows the pattern of IGFBP-3 secretion. The lowest IGFBP-2 secretion was found by explants with the highest rate of P53 protein IHC positive cells (80–100%) [4.02 ± 1.6 ng/10 mg of tissue]. The secretion of IGFBP-2 was higher by explants with 50–79.9% of P53 protein IHC positive cells [8.01 ± 1.8 ng/10 mg of tissue vs 4.02 ± 1.6 ng/10 mg of tissue; $p<0.05$]. The secretion of IGFBP-2 was the highest by explants with a ratio of P53 IHC positive cells lower than 50% [20.8 ± 1.8 ng/10 mg of tissue; 20.2 ± 2.2 ng/10 mg of tissue; 24.5 ± 2.6 ng/10 mg of tissue respectively in 0%; 0–9.9%; 10–49.9% of cells with positive IHCR for P53 protein (Figure 4).

No correlation was noted between the local secretion of PRL and the ratio of cells with positive IHCR for P53 protein (26.0 1.6; 24.41.4; 26.6 1.6p.; 25.71.5 ng/10 mg tissue respectively in 0–9.9%; 10–49.9%; 50–79.9% and 80–100% of cells IHC positive for P53 protein vs. 23.5 1.3 ng/10 mg tissue in explants with lack of IHC positive reaction for P53 protein cells (Figure 5).

DISCUSSION

The current results suggest no correlation between basic prolactin secretion by breast cancer explants and the level of the overexpression of P53 protein. Features like aggressiveness of the breast tumor, early disease relapse, metastases, patients with node negative breast cancer who have poor overall survival – also linked with increased level of P53 protein overexpression – were associated with hyperprolactinemia. However, there is limited direct evidence of the relationship between local prolactin secretion by breast malignant tissue and overexpression of p53. Fritsche *et al.* found that prolactin induction of Stat 5 mediated transcription was

repressed by P53 (Fritsche *et al.* 1998). Therefore, this makes the discussion of our results concerning the relationship between P53 overexpression and local prolactin secretion difficult.

On the other hand, results of the present study clearly showed a distinct difference in basic secretion of hGH and IGF-I by human breast cancer explants in relation to the ratio of cells with positive immunohistochemical reaction for P53 protein. Basic hGH secretion by breast cancer explants – having no cells with positive IHCR for P53 protein – was three times lower compared to those with overexpression of P53 protein. This observation may explain the clinically known phenomenon of poor prognosis for patients with high level of P53 protein overexpression. Wild-type P53 is a tumour suppressor gene that plays a central role in maintaining the genetic integrity of the cell by preventing cells with damaged DNA from further proliferation. Breast cancer cells that lack a wild-type functional form of P53 protein, do not undergo apoptosis. One of the features of hGH is its antiapoptotic activity. Specific P53 gene mutations have been associated with poor prognosis in breast cancer patients. According to our present observation, the growth of malignant tissue with a low level of p53 gene control (high level of overexpression) seems to be powered by locally secreted hGH. Mutations in the P53 gene or overexpression of P53 protein is also of value in the prediction of beneficial effects of adjuvant radiotherapy or chemotherapy in breast cancer. These types of treatment reduce the production of hGH. This may further support the role of hGH in the development of breast cancer.

Elledge *et al.* showed that the patient with a high ratio of cells immunohistochemically positive for P53 protein had shorter survival time compared to patients with lack of or low ratio of such cells (Elledge *et al.* 1994). In cancer cells that bear a mutant p53, this protein is no longer able to control cell proliferation, which results in inefficient DNA repair and the emergence of genetically unstable cells. The lack of control of proliferation may result from an increased secretion of hGH.

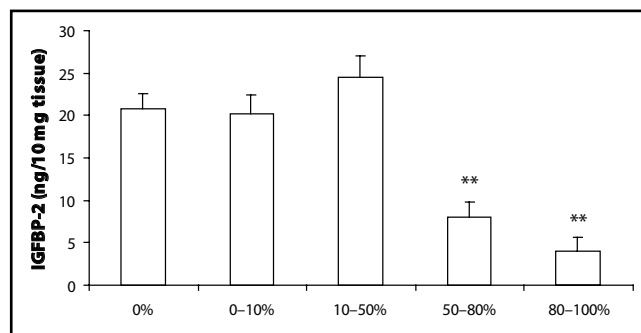


Fig. 4. Secretion of IGFBP-2 by breast cancer explants in relation to ratio of cells with showing positive immunohistochemical reaction (IHCR) to P53 protein (** $p < 0.05$).

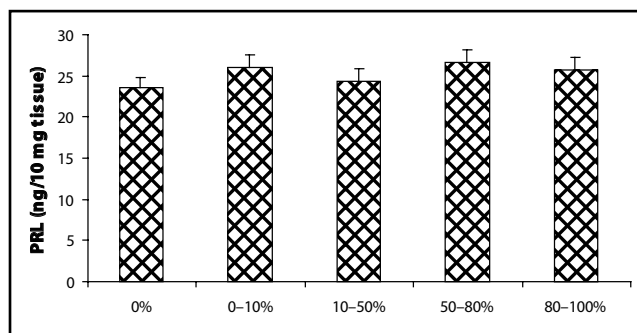


Fig. 5. Secretion of prolactin by breast cancer explants in relation to ratio of cells with showing positive immunohistochemical reaction (IHCR) to P53 protein.

There is a growing body of evidence supporting that locally produced IGF-I in breast tissue mediates the GH stimulated growth of breast tumours and, therefore, it is suggested to be one of the breast cancer development risk factors. On the contrary, the role of circulating IGF-I in breast cancer development remains uncertain. Our previous study showed hGH stimulation of local IGF-I secretion by human breast cancer explants. The disruption of normal interaction between P53 and IGF-mediated signal pathways can accelerate mammary tumorigenesis. IGFs have been shown to protect cells from apoptosis through an IGF-I receptor mediated cell survival pathway (Werner *et al.* 1996). Both the effects of a decrease in the number of IGF-receptors causing massive apoptosis and the overexpression of IGF receptors protecting cells from apoptosis have been demonstrated in vivo. A mutant version of P53 protein, commonly associated with malignant states, has been shown to derepress the IGF receptor promoter, with ensuing mitogenic activation by locally produced or circulating IGFs (Werner *et al.* 1996). The inability of mutant tumor suppressors to repress IGF-I receptor gene expression may result in increased levels of IGF-I receptor concentration and IGF binding which may lead to a reduction in apoptosis and enhanced survival capacity of malignant cells.

Takahashi & Suzuki found that growth stimulation of MCF-7 breast neoplastic cells by IGF-I is accompanied by tyrosine phosphorylation and nuclear exclusion of p53 (Takahashi & Suzuki 2003). Heron-Milhavet and Le Roitch showed that after 4-nitroquinoline 1-oxide-induced DNA damage, IGF-I induced exclusion of the p53 protein from the nucleus and led to its degradation in the cytoplasm, whereas p53 mRNA was unaffected. It is a novel role for IGF-I in the regulation of the MDM2/p53/p21 signaling pathway during DNA damage (Heron-Milhavet & Le Roitch 2002).

It may be hypothesized that the change in local secretion of hGH and IGF-I is linked to the change in ratio of cells with positive IHCR for P53 protein. However, our current observation shows no significant change

in basic secretion of hGH and IGF-I in correlation with the increase in ratio of cells with positive IHCR for P53 protein. The significant change was noted only between explants with and without cells with positive IHCR for P53 protein. In our present study, the basic secretion of IGF-I by explants lacking cells with positive IHCR for P53 protein is higher compared to those with overexpression of P53 protein. This result mirrors the pattern of hGH secretion by the same explants. Taking into account the well-known endocrine IGF-I/hGH negative feedback pathway, our present results seem to be plausible.

Hollowood *et al.* showed an auto/paracrine loop between IGFBP-3, one of components of IGF axis, and p53, which may provide control necessary to maintain normal tissue (Hollowood *et al.* 2000). IGFBP-3 induction by P53 constitutes a new means of cross talk between the P53 and IGF axis. It may also suggest the ultimate function of IGFBP-3 as a protective factor against the potentially carcinogenic effects of hGH and IGF-I (Grimberg 2000).

CONCLUSIONS

Our present data found that the influence of overexpression of P53 protein seems to be similar on local secretion of both IGFBP-3 and IGFBP-2. Our results showed an inverse relation between the ratio of cells with positive IHCR for P53 protein in breast cancer explants and secretion of IGFBP-2 and IGFBP-3. We also found the gradual decrease in secretion of these binding proteins with the increased ratio of cells immunohistochemically positive for P53 protein. This phenomenon was not clearly seen in regard to hGH and IGF-I secretion. A decrease in IGFBP-3 and IGFBP-2 local secretion increases the level of free IGF-I that is available to its receptors. Observation by Werner *et al.* and Webster *et al.*, that P53 regulates the expression of IGF-I-IR and IGFBP-3 suggests that there is a p53/IGF axis, whereby the increase in wild type P53 by DNA damage may induce apoptosis in part by down-regula-

tion of the antiapoptotic effects of IGF-I (Werner *et al.* 1996; Webster *et al.* 1996).

During mammary gland development, IGFBP-2 is expressed in the breast epithelial cells, while IGF-I is expressed in neighbouring stromal cells. IGFBP-2 is associated with apoptosis in the mammary gland at that stage. The exposure of breast cancer cell line Hs578T (which does not produce its own IGFBP-2) to exogenous IGFBP-2, increased p53 gene expression and showed the independence of IGF-I pro-apoptotic activity of IGFBP-2. Our recent results, which indicated the protective role of IGFBP-3 local secretion by breast cancer explants follows the observation of Grimberg (Grimberg 2000). Moreover, we also linked the changes in simultaneous, local secretion of IGFBP-3 and IGF-I, with tissue sex steroid receptor phenotype.

In conclusion, the presented data supports the hypothesis of the P53/hGH/IGF-I/IGFBP axis in breast cancer tissue. Furthermore, it also provides more evidence supporting the presence of local tissue hGH/IGF-I but not P53/PRL/IGF-I loop. These results should be considered preliminary, pending confirmation by other studies.

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